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Report

A Role for PtdIns(4,5)P_2 and PIP5Kα in Regulating Stress-Induced Apoptosis

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Summary

The phosphoinositide phosphatidylinositol 4, 5-bisphosphate (PtdIns(4,5)P_2) is essential for many cellular processes and is linked to the etiology of numerous human diseases [1–4]. PtdIns(4,5)P_2 has been indirectly implicated as a negative regulator of apoptosis [5–9]; however, it is unclear if apoptotic stimuli negatively regulate PtdIns(4,5)P_2 levels in vivo. Here, we show that two apoptotic-stress stimuli, hydrogen peroxide (H_2O_2) and UV irradiation, cause PtdIns(4,5)P_2 depletion during programmed cell death independently of and prior to caspase activation. Depletion of PtdIns(4,5)P_2 is essential for apoptosis because maintenance of PtdIns(4,5)P_2 levels by overexpression of PIP5Kα rescues cells from H_2O_2-induced apoptosis. PIP5Kα expression promotes both basal and sustained ERK1/2 activation after H_2O_2 treatment, and importantly, pharmacological inhibition of ERK1/2 signaling blocks PIP5Kα-mediated cell survival. H_2O_2 induces tyrosine phosphorylation and translocation of PIP5Kα away from its substrate at the plasma membrane, and both are dependent upon the activity of c-src family kinases. Furthermore, constitutively active c-src enhances tyrosine phosphorylation of PIP5Kα in vivo and is sufficient for the translocation of PIP5Kα away from the plasma membrane. These observations demonstrate that certain apoptotic stimuli initiate an essential signaling pathway during cell death, and this pathway leads to caspase-independent downregulation of PIP5Kα and its product PtdIns(4,5)P_2.

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Results and Discussion

H_2O_2 and UV Irradiation Trigger PtdIns(4,5)P_2 Depletion during Apoptosis

Oxidative-stress-induced apoptosis has been linked to Alzheimer's disease and cardiac infarction [10, 11] and can negatively regulate the PKB cell-survival pathway. We therefore postulated that H_2O_2 may negatively regulate PtdIns(4,5)P_2 levels during cell death. In HeLa cells, 600 μM H_2O_2 reproducibly caused apoptosis, which was blocked by the caspase inhibitor ZVAD-fmk (Figure 1A).

A GFP fusion of the PH domain of PLCδ1 (GFP-PH^{PLC}) was used as an in vivo PtdIns(4,5)P_2 probe [12, 13]. GFP-PH^{PLC} was concentrated at the plasma membrane in untreated cells (Figure 1B, left panel); however, 600 μM H_2O_2 caused translocation of GFP-PH^{PLC} from the plasma membrane, indicating PtdIns(4,5)P_2 depletion (Figure 1B, middle and right panels; see also Movie S1 in the Supplemental Data available online). PtdIns(4,5)P_2 levels did not recover after prolonged stimulation with H_2O_2 (1 hr) as indicated by the fact that GFP-PH^{PLC} remained diffusely spread throughout the cytosol (Figure 1B, right panel; see also Movie S1). To monitor PtdIns(4,5)P_2 depletion with increased temporal resolution, we used a fluorescence resonance energy transfer (FRET)-based assay [14]. Neurokinnin A (NKA)-mediated stimulation of phospholipase C (PLC) activity caused a transient decrease in PtdIns(4,5)P_2, which recovered to near basal levels within 2 min. Conversely, H_2O_2 treatment caused a sustained decrease in the FRET ratio, indicating prolonged (1 hr poststimulation) PtdIns(4,5)P_2 depletion. Complete activation of PLC by ionomycin and extracellular Ca^{2+} addition failed to further decrease the FRET ratio, indicating that levels of PtdIns(4,5)P_2 in the plasma membrane had already been significantly depleted (Figure 1C). These experiments demonstrate that the PtdIns(4,5)P_2 pool monitored by GFP-PH^{PLC} (which is sensitive to agonist stimulation) is depleted upon H_2O_2 stimulation. Because it has been suggested that GFP-PH^{PLC} also monitors intracellular Ins(1,4,5)P_3, we confirmed our imaging and FRET studies by metabolically labeling cells with [^{32}P]-orthophosphate to monitor phosphoinositide levels. In good agreement with our previous data, H_2O_2 markedly decreased PtdIns(4,5)P_2 labeling compared with that in untreated controls (Figure 1D), thereby confirming that apoptotic concentrations of H_2O_2 deplete PtdIns(4,5)P_2 levels.

As previously observed, both PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 labeling were increased after H_2O_2 treatment [15, 16] (Figure 1D). The increase in PtdIns(3,4,5)P_3 was transient, which we confirmed by using a monomeric RFP fusion of the PH domain of Grp1 (mRFP-PH^{Grp1}) as an in vivo PtdIns(3,4,5)P_3 probe [17]. This probe, which is predominantly cytosolic, was transiently recruited to the plasma membrane after treatment with 600 μM H_2O_2 (Figure S1A). In contrast, the increase in PtdIns(3,4)P_2 labeling was sustained (Figure 1D),

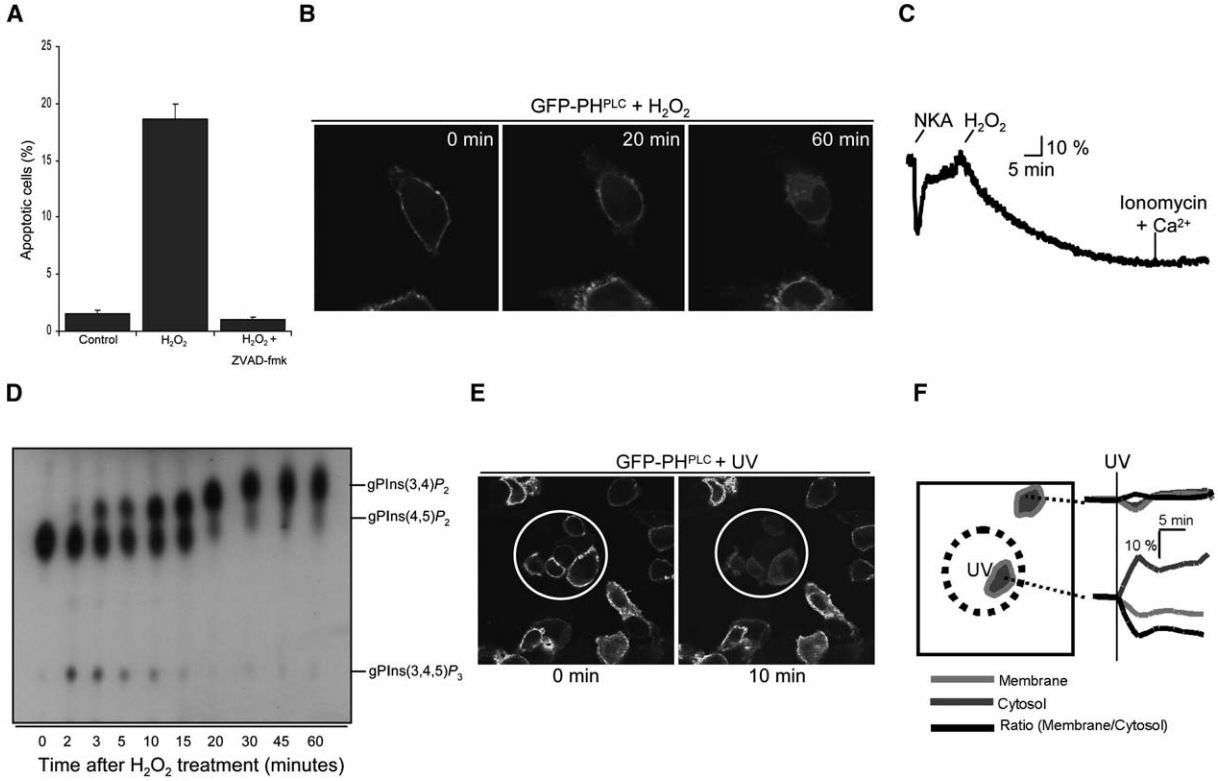

Figure 1. PtdIns(4,5)P2 Levels Are Depleted in Response to Oxidative Stress and UV Irradiation

(A) HeLa cells transfected with GFP-histone2B were left untreated (control) or were stimulated with 600 μM H_2O_2 in the presence or absence of 50 μM ZVAD-fmk as indicated for 24 hr, and the number of apoptotic nuclei was calculated (see the Supplemental Experimental Procedures). Data shown are representative of three different experiments. Error bars indicate standard deviation of triplicate samples.

(B) Cells grown overnight on glass coverslips were transfected with GFP-PH^{PLC} and stimulated with 600 μM H_2O_2. Confocal images were taken every minute for 60 min. Shown are representative images of cells prior to stimulation at 0 min, 20 min, and 60 min poststimulation.

(C) HeLa cells were transfected with YFP and CFP chimeras of the PH domain from PLCδ1 and the NKA receptor. Fluorescence data were captured with a wide-field microscope, and FRET changes were determined by calculation of the ratio of the CFP and YFP fluorescence [14]. Responsiveness of each cell was assessed by NKA addition. Cells were then treated with 600 μM H_2O_2, and the fluorescence ratio was monitored. Sixty minutes after stimulation, ionomycin and Ca^{2+} were added to maximally activate phospholipase C. The points of NKA, H_2O_2, ionomycin, and Ca^{2+} addition are indicated. Shown is a representative FRET trace of many independent experiments.

(D) Cells were either kept as controls (time = 0) or stimulated with H_2O_2 for the time indicated. The positions of HPLC-authenticated standards of gPIns(4,5)P_2, gPIns(3,4)P_2, and gPIns(3,4,5)P_3 are indicated.

(E) HeLa cells grown overnight on glass coverslips were transfected with GFP-PH^{PLC}. Cells were stimulated with UV irradiation for 30 s. By closure of the field diaphragm, only the center cells (within the white circle) were exposed to UV irradiation. Cells were imaged by confocal microscopy for 10 min with images being recorded every 30 s. Shown are representative images of cells prior to stimulation (left panel) and 10 min after UV irradiation (right panel).

(F) The cartoon highlights two analyzed cells for which the intensities of the fluorescence of the membrane (light-gray line) and the cytosol (dark-gray line), as well as the membrane/cytosol ratio (black line), were plotted in time. The graphs for each cell are indicated via a dashed line. Note the drop in the membrane/cytosol ratio of the cell within the circle (indicated by the dotted line) after UV irradiation and that the membrane/cytosol ratio of the cell outside the circle remains constant. The scale-bar signal shows the percent deviation from the baseline ratio value. Time and the point of UV irradiation are indicated.

suggesting that H_2O_2 promotes PtdIns(3,4)P_2 synthesis in the absence of or at the expense of PtdIns(3,4,5)P_3. Other apoptotic-stress stimuli such as UV irradiation also triggered rapid (occurring within 10 min) depletion of PtdIns(4,5)P_2 (Figures 1E and 1F). Because apoptosis by H_2O_2 and UV irradiation are both caspase dependent and because human PIP5Kα, which synthesizes PtdIns(4,5)P_2, was reported to be inactivated by caspase cleavage [9], we considered the possibility that PtdIns(4,5)P_2 depletion may occur downstream of caspase activation. Although ZVAD-fmk attenuated H_2O_2-induced apoptosis (Figure 1A), the caspase inhibitor failed to block PtdIns(4,5)P_2 depletion by either H_2O_2 (Figure 2A) or UV irradiation (Figure 2B). These data demonstrate that apoptotic-stress stimuli deplete PtdIns(4,5)P_2 levels independently of caspase activation.

PIP5Kα Expression Attenuates Apoptosis by H_2O_2 and UV Irradiation

To determine the importance of PtdIns(4,5)P_2 depletion during H_2O_2-dependent apoptosis, we attempted to block cell death by overexpressing PIP5K. Expression of murine PIP5Kα elevated PtdIns(4,5)P_2 levels by 1.5- to 2-fold (Figure 2C) and, after H_2O_2 stimulation, enabled the maintenance of cellular PtdIns(4,5)P_2 levels equivalent to those observed in untreated control cells (Figure 2C). Importantly, expression of murine PIP5Kα

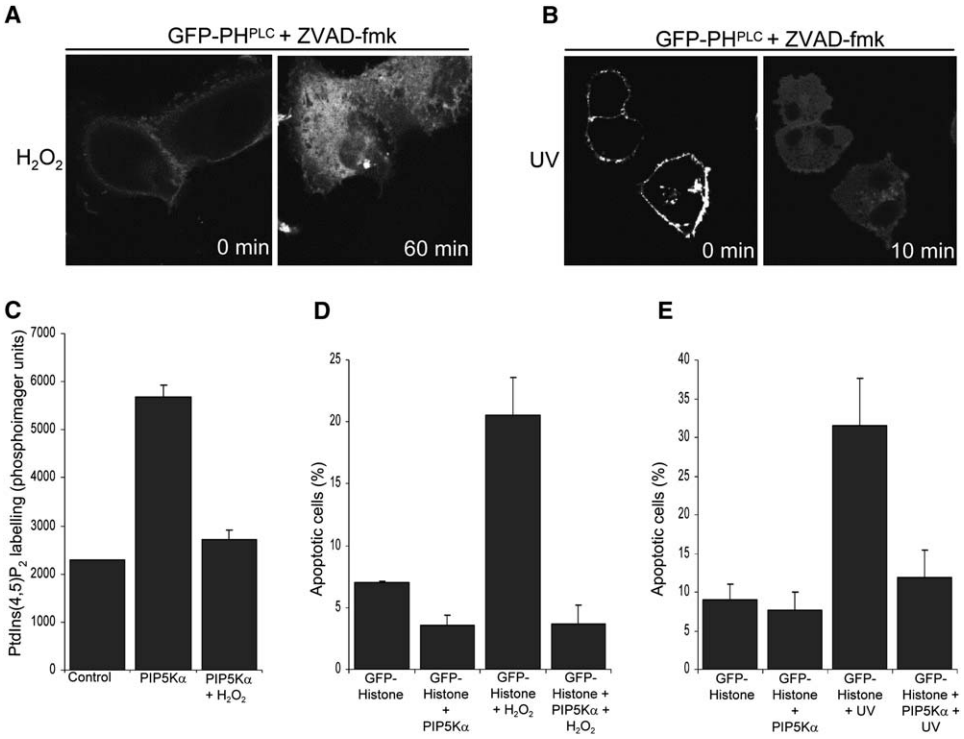

Figure 2. Overexpression of PIP5Kα Attenuates Oxidative-Stress- and UV-Irradiation-Induced Apoptosis

(A) HeLa cells transiently expressing GFP-PH^{PLC} were incubated with 50 μM (final concentration) ZVAD-fmk for 1 hr. Cells were then treated with 600 μM H_2O_2 and imaged for 60 min, with images being recorded every minute.

(B) Same as (A); however, cells were stimulated with UV irradiation for 30 s. Cells were then imaged for 10 min, with images being recorded every 30 s.

(C) HeLa cells transfected with either GFP-histone (control) or GFP-PIP5Kα (PIP5K) were metabolically labeled with [^{32}P]-orthophosphate and where indicated were treated with 600 μM H_2O_2 for 60 min, after which phospholipids were extracted and analyzed by thin layer chromatography (TLC). Plotted are the resulting PtdIns(4,5)P_2 levels determined by TLC and phosphoimager analysis. Error bars display standard deviation of triplicate samples. This graph is typical of three independent experiments.

(D) HeLa cells were transfected with various constructs (as indicated). Cells were stimulated with 600 μM H_2O_2 for 24 hr, and the number of apoptotic cells displaying fragmented nuclei was plotted as a percentage of the total number of transfected cells. Data shown are representative of three independent experiments. Error bars display standard deviation of triplicate samples.

(E) Same as (D); however, cells were instead irradiated with UV (180 mJ/cm^2) and incubated for a further 24 hr prior to being fixated and stained with Hoechst 33258. The data shown are representative of three independent experiments. Error bars display standard deviation of triplicate samples.

blocked apoptosis by H_2O_2 in HeLa cells (Figure 2D). The rescue of cells from apoptosis was not limited to H_2O_2 treatment because PIP5Kα overexpression also attenuated UV-irradiation-induced apoptosis (Figure 2E).

To further establish a causal relationship between PtdIns(4,5)P_2 depletion and apoptosis, we utilized a genetic approach to perturb plasma-membrane PtdIns(4,5)P_2 levels. A constitutively active version of Gq (Gαq*) can activate phospholipase C, leading to a depletion of PtdIns(4,5)P_2 levels and promotion of caspase-dependent apoptosis [6]. In HeLa cells expressing Gαq*, GFP-PH^{PLC} was diffusely spread throughout the cell (Figure S2A, left and right panels), indicating that plasma membrane PtdIns(4,5)P_2 levels were markedly depleted. Quantitative analysis revealed a 5-fold decrease in the membrane-cytosol ratio of GFP-PH^{PLC} (Figure S2B). In line with a role for PtdIns(4,5)P_2 depletion in apoptosis, we found that overexpression of murine PIP5Kα blocked Gαq*-induced apoptosis in HeLa cells (Figure S2C).

PIP5Kα Protects Cells from H_2O_2-Induced Apoptosis via ERK1/2

Previous studies showed that caspase activation in vitro could be inhibited by the addition of PtdIns(4,5)P_2. The same study showed that PIP5K overexpression inhibited TNFα-induced apoptosis, which the authors suggested occurred by caspase inhibition [9]. We detected no differences in H_2O_2-induced caspase activation between control cells and cells overexpressing murine PIP5Kα, indicating that the mechanism by which murine PIP5Kα promotes cell survival is not caspase inhibition (data not shown). Because PIP5Kα synthesizes PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in vivo [15], we examined whether PIP5Kα-mediated survival was linked to PKB activation [18]. Whereas expression of constitutively active phosphoinositide 3-kinase (CA-PI3-K) potently activated PKB, no such stimulation was observed by the overexpression of PIP5Kα (Figure S3A). Insulin, EGF, and CA-PI3-K promoted phosphorylation of endogenous PKB at residues serine 473 and threonine

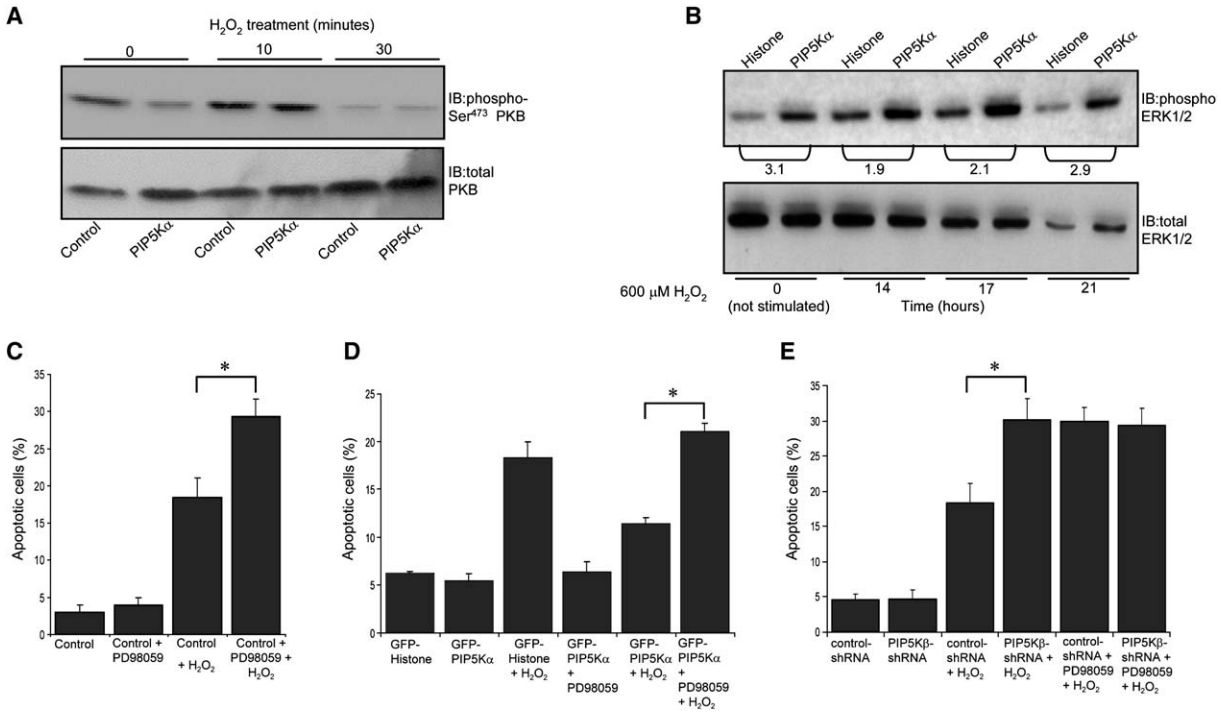

Figure 3. Activation of ERK1/2 Is Important in PIP5Kα-Mediated Attenuation of Stress-Induced Apoptosis

(A) Cells were transfected with either GFP-histone (control) or GFP-PIP5Kα and treated with H_2O_2 for the times indicated, and PKB activation was determined with phospho-serine 473 antibodies.

(B) HeLa cells were transfected with the constructs indicated. Sixteen hours after transfection, cells were treated with 600 μM H_2O_2 in the presence of serum for the times indicated; cells were then lysed, and ERK1/2 activation was ascertained with phospho-specific antibodies. Phospho-ERK signals were quantitated with densitometry, and values were normalized to the total ERK1/2 levels. Numbers underneath the upper panel denote the n-fold difference after normalization between the histone control and PIP5Kα samples at each time point.

(C) Where indicated, cells were treated with 20 μM PD98059 prior to stimulation with 600 μM H_2O_2 for 24 hr and the percentage of cells that underwent apoptosis was determined. Plotted graphically are the mean values of triplicate samples. The data shown are representative of three independent experiments. Error bars display the standard deviation of triplicate samples. A Student's t test was used to obtain p values of <0.02 (*) between the two samples indicated.

(D) HeLa cells were transfected with various constructs and pretreated with 20 μM PD98059 prior to stimulation with 600 μM H_2O_2 for 24 hr (as indicated). The number of transfected apoptotic cells displaying fragmented nuclei was plotted as a percentage of the total number of transfected cells (triplicate samples) and are representative of three independent experiments. Error bars display standard deviation of triplicate samples. A Student's t test was used to obtain p values of <0.001 (*) between the two samples indicated.

(E) HeLa cells stably expressing either control or PIP5Kβ-shRNA were treated with 600 μM H_2O_2 for 24 hr. Cells were also treated with 20 μM PD98059 prior to H_2O_2 stimulation where indicated. The percentage of apoptotic cells was determined. Plotted graphically are the mean values of triplicate samples and are representative of three independent experiments. Error bars display standard deviation of triplicate samples. A Student's t test was used to obtain p values of <0.02 (*) between the two samples indicated.

308 phosphorylation; however, PIP5Kα failed to promote phosphorylation of either amino acid (Figure S3B). Because H_2O_2 triggered PtdIns(4,5)*P*$_2$ depletion, it was possible that the maintenance of PtdIns(4,5)P_2 levels by PIP5Kα overexpression prolonged PKB activation after H_2O_2 treatment. H_2O_2 transiently activated PKB (Figure S3C); however, PIP5Kα failed to prolong PKB Ser-473 phosphorylation after H_2O_2 treatment (Figure 3A).

Members of the MAP kinase (MAPK) family of stress-activated kinases are linked with the regulation of cell survival. Although MAPK family members p38 MAPK and c-Jun N-terminal kinase (JNK) appear to play a role in regulating stress-induced apoptosis, previous reports have established ERK1/2 signaling as a critical protection mechanism against H_2O_2-mediated apoptosis [19, 20]. To determine whether PIP5Kα promoted cell survival via ERK1/2 signaling, we examined ERK1/2 phosphorylation in cells expressing PIP5Kα before and after treatment with H_2O_2. PIP5Kα overexpression led to a 3-fold increase in ERK1/2 phosphorylation compared with the basal condition (time point 0, Figure 3B). H_2O_2 induced ERK1/2 phosphorylation at short times (0–2 hr); however, no obvious differences between control and PIP5Kα-overexpressing cells were observed (data not shown). In contrast, after long-term H_2O_2 treatment (14–21 hr), ERK1/2 phosphorylation was greater in PIP5Kα-overexpressing cells than in control samples (Figure 3B). We also observed a decrease in total ERK1/2 levels after long-term treatment with H_2O_2. If PIP5Kα attenuated H_2O_2-induced apoptosis through the activation of ERK1/2 signaling, we would have expected that inhibition of ERK1/2 signaling would block this rescue. Pretreatment of HeLa cells with PD98059, a specific MEK-activity inhibitor that blocks ERK1/2 signaling, sensitized cells to H_2O_2-induced apoptosis (Figure 3C). Importantly, incubation with PD98059 prior to H_2O_2 treatment inhibited apoptotic rescue by PIP5Kα in HeLa cells (Figure 3D), indicating that ERK1/2

signaling was important for the PIP5K-dependent survival signal. It should be noted that PD98059 does not affect PtdIns(4,5)P_2 levels in vivo (data not shown). Because PIP5Kα overexpression protected HeLa cells from apoptosis, we hypothesized that PIP5K knockdown would sensitize cells toward H_2O_2-induced apoptosis. Therefore, we generated cell lines stably expressing either control-shRNA (empty vector) or human PIP5Kβ-shRNA (human PIP5Kβ is the homolog of murine PIP5Kα) (Figure S4). Compared to control-shRNA cells, those stably expressing PIP5Kβ-shRNA were more sensitive to H_2O_2-induced apoptosis (Figure 3E). Pretreating PIP5Kβ-shRNA cells with PD98059 did not further augment H_2O_2-induced apoptosis, indicating that the survival advantage conferred by PIP5Kβ is likely mediated via ERK1/2 activity (Figure 3E), and incubating PIP5Kβ-shRNA cells with PD98059 did not affect basal apoptosis levels (data not shown). Together, these data suggest that murine PIP5Kα (human PIP5Kβ) prevents apoptosis via ERK1/2 survival signaling. Furthermore, these data indicate that PtdIns(4,5)P_2 regulates ERK1/2 activation. How PtdIns(4,5)P_2 regulates ERK1/2 activity is not clear but could involve phosphoinositide-mediated regulation of Raf or PLD activity, or both [21, 22].

H_2O_2 Attenuates PIP5K Activity during Apoptosis

Because we were unable to measure PtdIns(4,5)P_2 resynthesis after H_2O_2 treatment, we considered the possibility that PIP5K activity was inhibited after prolonged oxidative stress. A previous report [9] has shown that activated caspase-3 can cleave and inactivate human PIP5Kα (homolog of the murine PIP5Kβ). In silico analysis of other PIP5K isoforms (murine PIP5Kα, γ, or H) revealed no homologous caspase-cleavage sites, and we were unable to detect cleavage of murine PIP5Kα after 4 or 6 hr of H_2O_2 treatment, at which time point caspase 3 is activated (data not shown). We then tested whether H_2O_2 instead modulated PIP5Kα catalytic activity by assaying endogenous PIP5K activity from HeLa cells after in vivo treatment with 600 μM H_2O_2. Endogenous PIP5K activity was inhibited after a 20 min incubation with H_2O_2; however, the inhibition was transient (Figure 4A). Because inactivation of PIP5K activity by H_2O_2 was transient, it was unlikely to account for the sustained PtdIns(4,5)P_2 depletion observed after H_2O_2 treatment. Therefore, we examined other potential mechanisms for PIP5K downregulation.

PIP5K activity is enriched at the plasma membrane (Figure 4B, left panel). Because the PIP5K substrate PtdIns4*P* is membrane localized and previous reports have linked in vivo PIP5K activity to membrane localization [23–25], we tested whether H_2O_2 treatment altered murine PIP5Kα localization. Within 20 min of 600 μM H_2O_2 treatment, a GFP fusion of murine PIP5Kα (GFP-PIP5Kα) translocated from the plasma membrane to the cytoplasm in a manner reminiscent of the H_2O_2-induced translocation of GFP-PH^{PLC} (Figure 4B; see also Movie S2) and remained cytosolic even after 60 min. GFP-PIP5Kα translocation was also observed after UV irradiation (Figure 4C). In both cases, GFP-PIP5Kα did not return to the plasma membrane several hours after treatment (data not shown). It has been shown previously that a PIP5K mutant, which does not localize to the plasma membrane, does not efficiently elevate PtdIns(4,5)P_2 levels in vivo [23]. GFP-PIP5Kα translocation could therefore constitute a mechanism by which PtdIns(4,5)P_2 synthesis at the membrane could be attenuated. Simultaneous imaging of cells expressing both GFP-PIP5Kα and mRFP-PH^{PLC} (to visualize the PtdIns(4,5)P_2 levels) did not reveal a temporal difference in translocation of either protein (Figure S5). Because H_2O_2 and UV irradiation both activate PLC and PtdIns(4,5)P_2 hydrolysis [26], it is likely that a coordinated activation of PLC and inactivation of PIP5K may be the underlying mechanisms for stress-induced PtdIns(4,5)P_2 depletion. In our model, PtdIns(4,5)P_2 is depleted by PLC activity (or in concert with PtdIns(4,5)P_2 phosphatases). Under normal conditions, after PLC activation, PtdIns(4,5)P_2 is rapidly resynthesized by the action of PIP5K (as observed in Figure 1C). During H_2O_2-induced apoptosis, however, PtdIns(4,5)P_2 resynthesis is blocked by the targeted inactivation of PIP5K. PtdIns(4,5)P_2 levels therefore never recover, and the cell enters apoptosis.

PIP5Kα Translocation Is Dependent upon Src Kinase Activity

To define the events regulating the H_2O_2-dependent translocation of PIP5Kα, we performed an inhibitor screen to identify signaling components important for the translocation process. PP1 and PP2 (both specific inhibitors of src tyrosine kinases) were found to inhibit GFP-PIP5Kα translocation by H_2O_2 (Figures 4D and 4E), whereas SP600125 (inhibitor of JNK signaling), SB203580 (inhibitor of p38 MAPK signaling), ZVAD-fmk (caspase inhibitor), or wortmannin and LY294002 (PI3K inhibitors) did not inhibit GFP-PIP5Kα translocation (data not shown). We then tested whether PIP5Kα was tyrosine phosphorylated after H_2O_2 treatment. Indeed, in vivo GFP-PIP5Kα was tyrosine phosphorylated after H_2O_2 treatment. Importantly, PP1 and PP2 inhibited tyrosine of phoshorylation of GFP-PIP5Kα by H_2O_2 (Figure 4F), suggesting the involvement of src tyrosine kinases. To confirm whether activation of c-src alone mediates phosphorylation of PIP5Kα in vivo, we examined the phosphorylation status of GFP-PIP5Kα in cells expressing constitutively active c-src (CA c-src). Coexpression of CA c-src caused tyrosine phosphorylation of GFP-PIP5Kα in vivo (Figure 4G). C-src phosphorylates PIP5Kγ on amino acid Y644 in the C-terminal region [27]. This region is not present in murine PIP5Kα and β, and it has not been reported that c-src phosphorylates PIP5Kα or β. To establish whether c-src activation was sufficient for PIP5Kα translocation, we coexpressed CA c-src with murine PIP5Kα. CA c-src expression caused translocation of mRFP-PIP5Kα in vivo; this was evident by a significant decrease in the membrane/cytosol ratio of mRFP-PIP5Kα in CA c-src-expressing cells compared to control cells (mRFP-PIP5Kα alone) (Figures S6). We have not demonstrated that c-src directly phosphorylates PIP5Kα; however, we have shown that c-src activity is critical for the tyrosine phosphorylation of PIP5Kα after H_2O_2 treatment and that c-src activity alone can induce phosphorylation of PIP5Kα in vivo and translocation of PIP5Kα away from the plasma membrane. It would appear that regulation of the PIP5K family by c-src may be a general phenomenon.

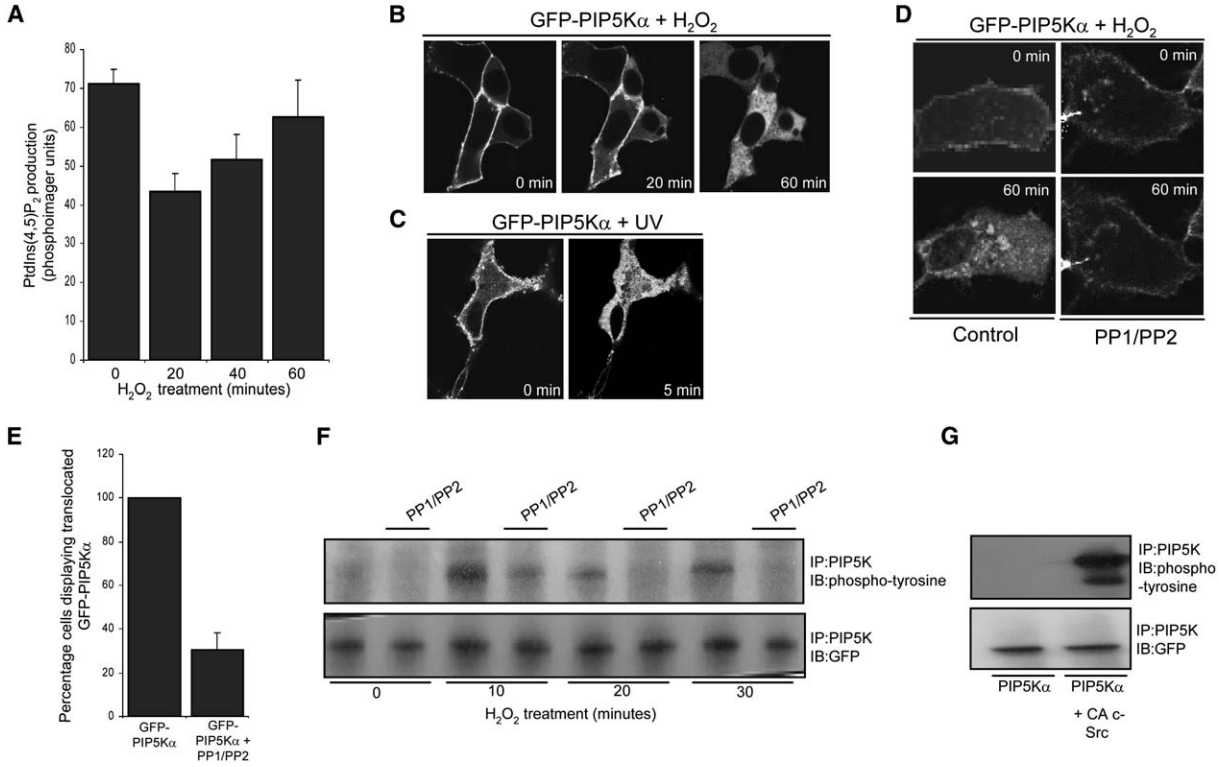

Figure 4. Oxidative Stress and UV Irradiation Induce the Delocalization of PIP5Kα from the Plasma Membrane through the Activation of Src Family Kinases

(A) HeLa cells were stimulated with 600 μM H_2O_2 for the times indicated. Cells were lysed, and endogenous PIP5K was immunoprecipitated via a polyclonal antibody. Immunoprecipitates were assessed for PIP5K activity with mixed micelles containing PtdIns4*P* as a substrate (see the Supplemental Experimental Procedures). Lipids were extracted and separated with TLC, and PtdIns(4,5)P_2 production was quantified with a phosphoimager. Plotted graphically are the mean values of triplicate samples. Error bars represent the standard deviation of the mean of triplicate samples.

(B) Shown are confocal images of the mid-section of cells expressing GFP-PIP5Kα. Cells were grown on glass coverslips overnight prior to transfection. Cells were stimulated with H_2O_2 and imaged live 16 hr after transfection. Shown are representative images of cells prior to stimulation and 20 and 60 min after stimulation with H_2O_2.

(C) Identical to (B) except cells were stimulated with UV irradiation and images were taken every 20 s. Shown is a representative image of cells prior to stimulation and 5 min after treatment with UV.

(D) Translocation of GFP-PIP5Kα was evaluated after treatment with 600 μM H_2O_2. Where indicated, cells were pretreated with 10 μM PP1 and 10 μM PP2 for 45 min prior to H_2O_2 treatment. Shown are representative images.

(E) Graphical representation of the percentage of cells displaying translocated GFP-PIP5Kα 1 hr after H_2O_2 treatment without or with PP1 and PP2 pretreatment. A total of 30 cells for each condition were scored for translocation 60 min after H_2O_2 treatment.

(F) HeLa cells expressing GFP-PIP5Kα were either maintained as controls or pretreated with 10 μM PP1 and 10 μM PP2 for 45 min prior to stimulation with 600 μM H_2O_2 for the times indicated. Cells were lysed, PIP5Kα was immunoprecipitated, and, after western blotting, nitrocellulose membranes were probed with antibodies against phospho-tyrosine (PY99 top panel) or GFP (bottom panel).

(G) HeLa cells were transfected with the constructs shown, and recombinant GFP-PIP5Kα was immunoprecipitated with PIP5K-specific antibodies and, after western blotting, nitrocellulose membranes were probed with antibodies against phospho-tyrosine (top panel) or GFP (bottom panel).

We propose that c-src is activated in response to H_2O_2 treatment and that its activation in turn mediates the tyrosine phosphorylation of PIP5Kα and causes translocation of PIP5Kα away from the plasma membrane. Maintenance of the PtdIns(4,5)P_2 level is certainly essential for viability, and we demonstrate that PIP5Kα overexpression can rescue cells from H_2O_2-induced apoptosis mediated by the activation of ERK1/2 signaling. In contrast, human PIP5Kβ knockdown sensitizes cells to H_2O_2-induced apoptosis. Furthermore, overexpression of PIP5Kα and subsequent elevation in PtdIns(4,5)P_2 levels can activate basal endogenous ERK1/2 signaling. These data identify a new link among PIP5Kα, PtdIns(4,5)P_2, and ERK1/2 regulation. In conclusion, certain apoptotic stimuli negatively regulate PtdIns(4,5)P_2 levels in vivo, and this constitutes an important early signaling step during programmed cell death.

Supplemental Data

Supplemental Data include Experimental Procedures, six figures, and two movies and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/18/1850/DC1/.

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